Send Orders for Reprints to reprints@benthamscience.net

 Current Pharmaceutical Design, **2013**, *19*, 5374-5383

The Culture-Repopulating Ability Assays and Incubation in Low Oxygen: A Simple Way to Test Drugs on Leukaemia Stem or Progenitor Cells

Maria Grazia Cipolleschi, Elisabetta Rovida and Persio Dello Sbarba*

Dipartimento di Patologia e Oncologia Sperimentali dell'Università degli Studi di Firenze e Istituto Toscano Tumori, viale G.B. Morgagni 50, 50134 Firenze, Italy

Abstract: The Culture-Repopulating Ability (CRA) assays is a method to measure *in vitro* the bone marrow-repopulating potential of haematopoietic cells. The method was developed in our laboratory in the course of studies based on the use of growth factor-supplemented liquid cultures to study haematopoietic stem/progenitor cell resistance to, and selection at, low oxygen tensions in the incubation atmosphere. These studies led us to put forward the first hypothesis of the existence *in vivo* of haematopoietic stem cell niches where oxygen tension is physiologically lower than in other bone marrow areas. The CRA assays and incubation in low oxygen were later adapted to the study of leukaemias. Stabilized leukaemia cell lines, ensuring genetically homogeneous cells and enhancing repeatability of results, were found nevertheless phenotypically heterogeneous, comprising cell subsets exhibiting functional phenotypes of stem or progenitor cells. These subsets can be assayed separately, provided an experimental system capable to select one from another (such as different criteria for incubation in low oxygen) is established. On this basis, a two-step procedure was designed, including a primary culture of leukaemia cells in low oxygen for different times, where drug treatment is applied, followed by the transfer of residual cell population (CRA assay) to a drug-free secondary culture incubated at standard oxygen tension, where the expansion of population is allowed. The CRA assays, applied to cell lines first and then to primary cells, represent a simple and relatively rapid, yet accurate and reliable, method for the pre-screening of drugs potentially active on leukaemias which in our opinion could be adopted systematically before they are tested *in vivo*.

Keywords: Hypoxia, stem cell cycling, stem cell niche, leukaemia stem cells, minimal residual disease, chronic myeloid leukaemia, BCR/Abl, resistance to Imatinib.

INTRODUCTION

Liquid haematopoietic cell cultures. About fifty years ago, information on clonogenic haematopoietic progenitors was obtained first by transplanting cells into lethally irradiated mice (*in vivo* clonal assays) [1, 2] and shortly after with data derived from cell cultures in semisolid medium (*in vitro* clonal assays) [3]. On the other hand, cultures of haematopoietic cells in liquid medium were also introduced as early as in 1966 [4-6]. In these cultures, later referred to as *short-term liquid cultures*, the addition of haematopoietic growth factors boosts the maximal expansion of cell population. About ten years later, *long-term liquid cultures* were developed where no exogenous growth factor is added and the relationship between maintenance of stem/progenitor cell potential and microenvironment could be addressed [7].

"Liquid-to-semisolid" cell transfer. Cell transfer at different times of incubation from (primary) liquid cultures to (secondary) clonal assays in semisolid medium [8] enables to monitor the kinetics of generation in liquid culture of Colony-Forming Cells (CFC) from more immature progenitors (thereby called *pre-CFC*). Cell transfer from cytokine-supplemented liquid cultures allows, in addition, to evaluate differences of response to cytokines between CFC and pre-CFC [9]. Although transfer to secondary clonal assays can be also done with cells rescued from primary colonies grown in semisolid cultures (*serial* clonal assays), the liquid-to-semisolid culture cell transfer is best to determine the overall pre-CFC content of a haematopoietic cell population. This is not only because this technique is less time-consuming and easier to execute, but also because it is unaffected by the experimental variability due to the resuspension and replating of cells rescued from arbitrarily chosen primary colonies.

*Address correspondence to this author at the Dipartimento di Patologia e Oncologia Sperimentali dell'Università degli Studi di Firenze e Istituto Toscano Tumori, viale G.B. Morgagni 50, 50134 Firenze, Italy;
Tel/Fax: +39 055 4598209; E-mail: persio@unifi.it

"Liquid-to-liquid" cell transfer. Methods based on both primary and secondary liquid cultures (liquid-to-liquid cell transfer) were also developed. An example can be found within a group of assays known as *Delta* assays because they measure the production of a number of clonogenic cells (base of the Delta) by a single progenitor (apex of the Delta), Delta being the symbol commonly used to draw the so-called *subpopulation diagrams* describing the haematopoietic regeneration hierarchy [10, 11]. Indeed, one of the most advanced versions of Delta assays is a cytokine-driven sequential dilution/expansion assay where cells grown in cytokine-supplemented liquid cultures are subjected weekly to dilution and complete change of culture medium. The cumulative generation of cells or CFC over 3-4 weeks of incubation is taken as a measure of the regenerative potential of the input cell population and of its pre-CFC content in particular [12, 13].

"Liquid-to-vivo" cell transfer. Finally, primary liquid cultures appeared the most convenient experimental approach when cultured cells were to be transferred to secondary stem/progenitor cell assays *in vivo* (liquid-to-*vivo* cell transfer) [14, 15], of either the clonal or the non-clonal classes [16]. Among the latter, the Marrow-Repopulating Ability (MRA) assays will be especially taken into consideration below.

LIQUID HAEMATOPOIETIC CELL CULTURES INCUBATED AT LOW OXYGEN TENSION

Cell transfer from primary cytokine-supplemented liquid cultures to secondary stem/progenitor cell assays was adopted in our laboratory to undertake studies of the metabolic regulation of haematopoiesis. We demonstrated first that pyruvate, the metabolite linking glycolysis to Krebs' cycle and cell respiration, reduces the expansion of haematopoietic cell populations, but not the generation of CFC *in vitro* [9]. Later, we addressed directly the effects of inhibition of cell respiration, by incubating haematopoietic cells at an oxygen concentration (1% O_2) markedly lower than that of the standard incubation atmosphere (air; 21% O_2). Different subsets of

1873-4286/13 $58.00+.00

murine clonogenic haematopoietic cells (as detected by the assays indicated in Fig. **1**) were shown to exhibit different behaviour with respect to the incubation atmosphere [14, 17]. It emerged that: (a) haematopoiesis is overall inhibited at reduced oxygen tension; (b) resistance of haematopoietic progenitors to low oxygen is the higher, the higher their level within the haematopoietic hierarchy; (c) short-term repopulating haematopoietic stem cells (HSC) are maintained in low oxygen better than in air, implying that (d) in low oxygen HSC are selected and enriched from haematopoietic cell populations. These data were extended in our laboratory to human cells [18, 19] and by others, ten years later, to long-term repopulating HSC [20]. Furthermore, Fig. **1** shows that incubation at low oxygen tension ensures not only the enrichment of HSC with respect to incubation in air, but even their (limited, but significant) expansion with respect to time 0, provided an appropriate combination of stem cell-active factors is added to cultures (right graph). Thus, it emerged that the regulative role of tissue oxygen tension on haematopoiesis is integrated with those of other environmental factors, such as cytokines [17].

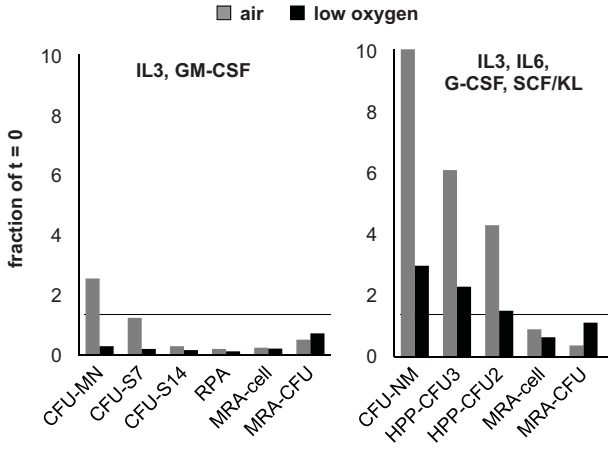

Fig. (1). Maintenance or expansion of different subsets of haematopoietic stem or progenitor cells in low oxygen.
Normal murine bone marrow cells were cultured in liquid medium containing the indicated cytokines and incubated in atmosphere at 1% oxygen (black) or in air (grey) for 5 (left graph) or 8 (right graph) days. Cells were then subjected to the indicated stem or progenitor cell assays: CFU-NM, neutrophil/monocyte colony formation *in vitro*; CFU-S7/14, spleen colony formation *in vivo*, read 7 or 14 days after transplantation; RPA, protection from death due to lethal irradiation; MRA, bone marrow repopulation; HPP, high proliferative potential colony formation *in vitro* (CFU3, less immature; CFU2, more immature). The addition of a combination of stem cell-active factors (right) enhanced, as expected, the yield of assays. Histograms represent the culture content of stem or progenitor cells expressed as percentages of the corresponding time 0 values (horizontal lines) and are means of at least three independent experiments. Recalculated from data reported in [14, 17].

THE "HYPOXIC" STEM CELL NICHE HYPOTHESIS

All above pointed to low oxygen tension as a relevant aspect of the environment where HSC are maintained, which Ray Schoefield had modelled and called *stem cell niche* [21]. On this basis, we put forward the first "hypoxic" HSC niche hypothesis, predicting that low oxygen tensions favour HSC maintenance and self-renewal while restraining clonal expansion and the parallel exhaustion of stem cell potential [14]. This hypothesis was shown to hold true by a number of later studies carried out by others [reviewed in 22, 23]. It is worth pointing out here that very low oxygen tensions represent a *physiological* feature of stem cell niche, implying that an environment which is "hypoxic" for the bulk of haematopoietic cells is actually "normoxic" for HSC. This issue is reviewed elsewhere [24, 25].

THE CULTURE-REPOPULATING ABILITY ASSAYS

The selective power of low oxygen tension on haematopoietic cell populations and its capacity to enrich liquid cultures with short term-repopulating HSC detectable *in vivo* (MRA assay) led to establish "liquid-to-liquid" culture cell transfer methods called the *Culture-Repopulating Ability* (CRA) assays [26], which, by analogy with Delta assays, can be defined as cytokine-driven sequential selection/expansion assays (Fig. **2**). The CRA assays estimate the stem cell (pre-CFC) potential contained within a primary liquid culture (LC1) at the end of its experimental manipulation by transferring LC1 cells to secondary liquid cultures (LC2) where the expansion of cell population is unrestrained, and the stem cell potential is thereby exploited following a further incubation. With this method, the effects of a selection treatment applied to LC1 (incubation in low oxygen) on the maintenance of stem cell potential at the end of LC1 are determined by transferring LC1 cells to non-selective LC2 (incubation in air). LC2 repopulation is determined by counting the total number of cells (CRA_{cell}), or determining the number of CFC (CRA_{CFC}) after transfer of LC2 cells to (tertiary) semisolid cultures and a further incubation therein (as appropriate for the CFC assay *in vitro*). It is evident from Fig. **2** that LC2 "is" the CRA assay, that represents indeed an *in vitro* adaptation of the MRA assay *in vivo*; this is relevant to the validation of CRA assays (see below). It is worth pointing out that, by analogy with MRA assays, CRA assays can be also applied to an unmanipulated cell population, such as that directly recovered from donor animals or routine cultures. This is done, for example, to get the control data necessary to measure percentage CRA maintenance in low oxygen, as shown in Tables **1** and **2**. However, the "liquid-to-liquid", LC1-to-LC2 cell transfer becomes necessary when one wants to test a drug on cells, which occurs in LC1. How the maintenance of stem cell potential in LC1 is calculated on the basis of LC2 repopulation is also explained below.

In the study where the CRA assays were designed [26], LC1 were incubated at low oxygen tension in the presence of a cytokine combination similar to that of Fig. **1**, left graph, for at least 7 days. Under these conditions, a one-log reduction of total cell number or CFC number with respect to time 0 of LC1 is obtained and the risk to "contaminate" LC2 with CFC or other proliferating still persisting in, and therefore imported from, LC1 is minimal. The purpose of CRA assays is, indeed, to evaluate the LC2 repopulation ability of pre-CFC (stem cells in particular) which may result resistant to the selective treatment applied to LC1. Thus, the above precaution yields a "cleaner" LC2 repopulation kinetics, thereby enhancing the accuracy of the estimate of stem cell potential rescued from LC1. However, standard CRA assays do not provide resolution between different functional subsets of the pre-CFC compartment which exhibit equal resistance to selection. Such a lack of resolution can be overcome by extending the duration of selective incubation of LC1 or treating LC1 with cell cycle-specific antiblastic drugs which may differently affect different functional subsets of the pre-CFC compartment (see below).

Table **1** summarizes the results of a number of studies, carried out by us and others, where the effects of incubation in low oxygen of cytokine-driven haematopoietic cell cultures on the maintenance of stem/progenitor cell potential were determined by CRA and/or MRA or $SCID_{mice}$-Repopulating Cell (SRC) assays [see table for references]. When CRA assays were used, this maintenance was estimated by the ratio of peak values reached in LC2 established with cells rescued from selective LC1 incubated in low oxygen to peak values reached in LC2 established with cells rescued from non-selective, air-incubated control LC1. Alternatively, to calculate the above ratio, cumulative LC2 repopulation (calculated as described in reference [27]) can be taken into consideration. However, when the two criteria were compared within the same study, they were found to generate equivalent results (compare with each other the two values in line 9 and in line 10). The data reported in Table **1**

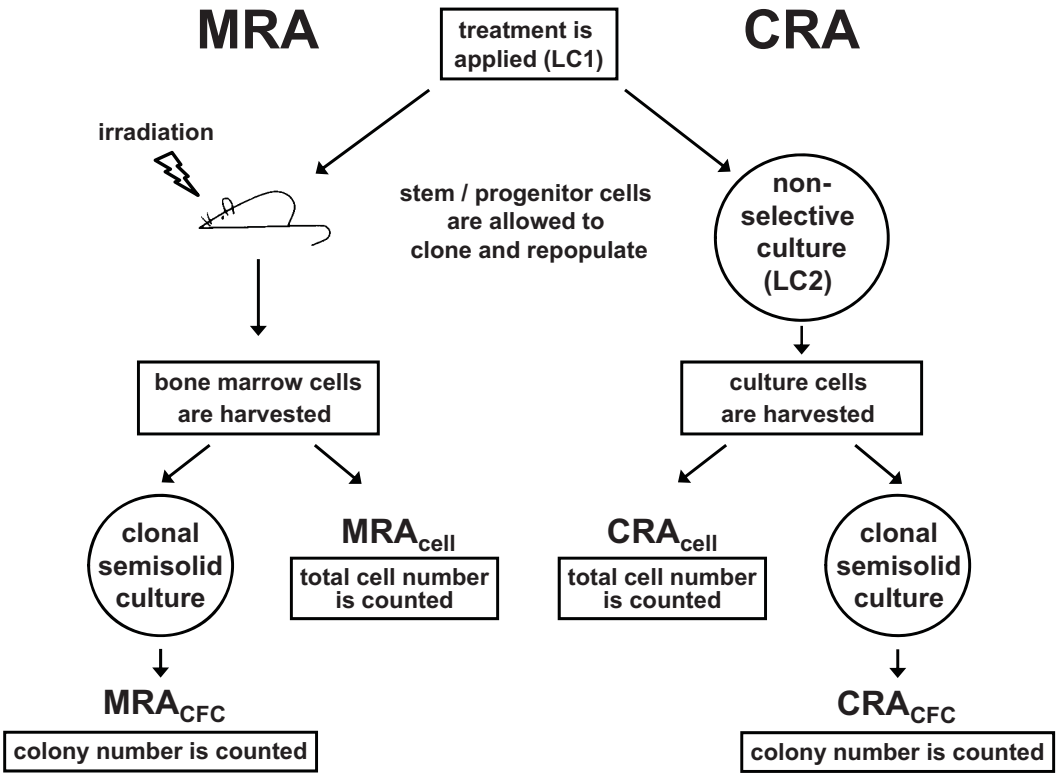

Fig. (2). The CRA and MRA assays.
Schematic representation of CRA and MRA assays and their relationship. Note that the CRA assays consist of the establishment of LC2 and subsequent phases. LC1, where treatment is applied to cells and the experiment actually takes place, is not technically part of CRA assay, as LC1 cells can be also assayed following transplantation *in vivo* (MRA).

led to the following main conclusions: (a) when carried out in parallel, CRA_{cell} and CRA_{CFC} assays yielded very similar results, respectively, to MRA_{cell} and MRA_{CFC} assays, which is the way CRA assays were validated (lines 5-8); (b) when the same cells and comparable cytokine combinations were used, both MRA and CRA assays were very consistent across independent studies in estimating the maintenance of stem/progenitor cell potential in low oxygen; (c) combinations of stem cell-active factors can push stem cell compartment to expand in low oxygen with respect to the beginning of incubation, as also shown in Fig. **1**, right graph.

THE QUESTION OF HSC CYCLING AT LOW OXYGEN TENSION AS ADDRESSED BY CRA ASSAYS

The finding that the stem cell compartment can expand in low oxygen [17] prompted us to evaluate by CRA assays the question of whether stem cells cycle in low oxygen. Treatment of LC1 with the cell cycle-specific antiblastic drug 5-fluoro-uracil (5FU) before the end of incubation (followed by transfer to LC2 of cells washed free of drug) resulted in the complete suppression of either CRA_{cell} or CRA_{CFC} in air-incubated LC1 and of CRA_{cell} in low-oxygen LC1. On the other hand, as far as CRA_{CFC} is concerned, 1/3 (~25% of the time-0 value) resulted 5FU-resistant, but 2/3 were 5FU-sensitive, apparently because they were cycling [26]. These results indicated that in low oxygen: (a) progenitors sustaining CRA are capable to cycle; (b) all progenitors sustaining CRA_{cell} are cycling; (c) progenitors sustaining CRA_{CFC} have the option between cycling and quiescence. Such a different behaviour is very well in keeping with our "hypoxic" stem cell niche hypothesis: progenitors sustaining CRA_{CFC} would represent HSC the maintenance of which is ensured in the core of niche; on the other hand, progenitors sustaining CRA_{cell} would be in charge of clonal expansion at the periphery of niche.

The issue of HSC cycling in low oxygen was deepened by replating into LC2 separately cells sorted on the basis of the number of replication cycles accomplished in LC1 (flow-cytometry following PKH26 labelling). CRA_{CFC} assay showed that, in low-oxygen LC1, stem cell potential is markedly enhanced in cells which have undergone one replication cycle, while it is rapidly lost when cycling is sustained beyond the first cycle. In air-incubated LC1, on the other hand, this loss occurs independently of the number of cycles accomplished [27]. Thus, incubation at low oxygen allows, but also restrains, HSC cycling, thereby steering cycling in favour of the maintenance of stem cell potential in HSC which just exited quiescence. In other words, self-renewal would be ensured immediately after the rescue of quiescent HSC to cycling in low oxygen. In this perspective, low oxygen would be a crucial factor in regulating stem cell compartments and represent the driving force of a dynamic, rather than static, stem cell maintenance within the stem cell niche.

EFFECTS OF LOW OXYGEN TENSION ON LEUKAEMIA CELLS AS DETERMINED BY CRA ASSAYS

The CRA assays were developed as simple short term-repopulating stem cell assays, with two main purposes in mind: (a) to overcome the problem of low probability of survival of transplanted animals during *in vivo* repopulation assays (unless the complex *competitive repopulation assay* is used) when rare stem cell subsets represent the experimental target; (b) to overcome the necessity of using, when human stem cells are to be tested, the expensive and often cumbersome *in vivo* repopulation assays based on xeno-transplantation. On this basis, the most interesting field of application of CRA assays appeared, since the very beginning, the study of human leukaemias, to determine in particular the drug sensitivity of Leukaemia Stem Cells (LSC). For solid neoplasias,

Table 1. Maintenance of Haematopoietic Stem/Progenitor Cell Potential in Low Oxygen.

See text and referenced papers for details and explanation. MRA or SRC data are ratios of values obtained by transplanting *in vivo* cells incubated in low oxygen to the corresponding values obtained transplanting non-cultured cells (Column 6) or cells incubated in air (Column 7). CRA data (Column 6) are usually ratios of peak values of LC2 (always incubated in air) established with cells transferred from LC1 at the end of incubation in low oxygen to peak values of control LC1 incubated in air; an exception to this criterion are the cases indicated by an asterisk, where CRA data are ratios of cumulative LC2 repopulation values (the integral of repopulation kinetics [27], instead of peak of repopulation); n.a., not assessed. Data printed in bold refer to previously unpublished calculations. In collaboration with Prof. Vincent Praloran and coworkers, Laboratoire d'Hématopoïèse Normale et Pathologique, Université Victor Segalen - Bordeaux-2, and Prof. Zoran Ivanović and coworkers, Etablissement Français du Sang - Aquitaine-Limousin, Bordeaux, France.

1		2	3	4	5	6	7	8
cells		cytokines in LC1	days in LC1 (% O_2)	time in LC2 (peak) or *in vivo*	assay	maintenance of stem cell potential		ref.
						low O_2 / t=0	low O_2 / air	
1	murine bone marrow	PWM-stimulated lymphocyte CM	5 (1.0)	14d	MRA_{cell}	0.23	0.81	[14]
2					MRA_{CFC}	0.74	1.36	
3	murine bone marrow	G-CSF, SCF/KL, IL6, IL11	8 (1.0)	14d	MRA_{cell}	0.63	0.70	[17]
4					MRA_{CFC}	1.12	2.95	
5	murine bone marrow	GM-CSF, IL3	14 (1.0)	14d	MRA_{cell}	0.18	n.a.	[26]
6					MRA_{CFC}	0.69		
7				9d	CRA_{cell}	0.12	n.a.	
8					CRA_{CFC}	0.76		
9	murine bone marrow	G-CSF, SCF/KL, IL6	8 (1.0)	10d	CRA_{CFC}	**1.25 (1.12*)**	n.a.	[27]
10		G-CSF, SCF/KL, IL6, IL3				**0.60 (0.55*)**		
11	CD34+ cord blood	G-CSF, SCF/KL, IL3, MGDF	7 (3.0)	14d	CRA_{CFC}	**1.86**	n.a.	[28]
12				28d		**6.25**		
13				8w	SRC	n.d.	**2.33**	
14	Lin- murine bone marrow	IL-3	10 (1.0)	10d	CRA_{CFC}	0.56	n.a.	[29]
15		IL-3, IL-6				0.78		
16	CD34+ cord blood	G-CSF, SCF/KL, IL3, TPo	10 (1.5)	14d	CRA_{CFC}	**1.24**	n.a.	[30]
17		G-CSF, SCF/KL, IL3, TPo (+MSC)				**2.61**		
18		G-CSF, SCF/KL, IL3, TPo		8w	SRC_{cell}	**0.65**	**1.86**	
19		G-CSF, SCF/KL, IL3, TPo (+MSC)				**5.75**	**4.96**	
20		G-CSF, SCF/KL, IL3, TPo			SRC_{CFC}	**0.50**	**2.53**	
21		G-CSF, SCF/KL, IL3, TPo (+MSC)				**1.05**	**1.18**	

indeed, other *in vitro* assays have been developed to monitor the maintenance of stem cell potential, such as the serial clonogenic assays where epithelial tumor-spheres are generated in liquid cultures and then tumor-sphere cells are transplanted into secondary liquid cultures [reviewed in 31].

The adaptation of CRA assays to study leukaemia cell populations was carried out using Murine Friend's Erythro-Leukaemia (MEL) cells and incubation at 0.1% O_2 to select stem cells [32]. This study led to a first set of conclusions: (a) a clonal leukaemia cell line is heterogeneous with respect to the cell functional phenotype and hierarchically organized like a normal haematopoietic population; (b) the MEL cell population includes the highly oxygen-dependent cell bulk and CFC, as well as pre-CFC resistant to/selected in low oxygen, which (c) efficiently and consistently repopulate LC2, as determined by CRA_{cell} assay (Fig. **3**, square); (d) maintenance at low oxygen is a peculiarity of stem cells as opposed to the rest of haematopoietic cell population, irrespective of whether they are normal or leukaemic.

The relationship of LSC cycling to their maintenance in low oxygen was then addressed following criteria similar to those used for HSC, *i.e.* by treating LC1 with 5FU close to the end of incubation and transferring cells washed free of drug to LC2. LC1 treatment with 5FU completely suppressed (Fig. **3**, circle) the LC2 repopulation driven according to the standard CRA_{cell} kinetics (Fig. **3**, square). However, 5FU-treated LC1 cells repopulated LC2 after a long lag-phase (*extended*CRA_{cell}), following a kinetics (Fig. **3**, cir-

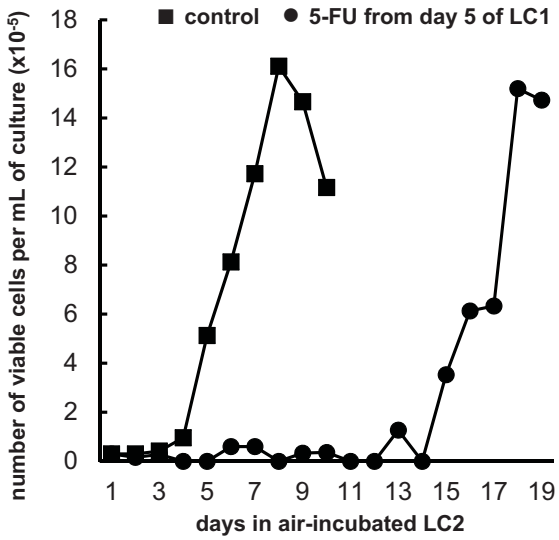

Fig. (3). Maintenance of Friend's erythroleukaemia stem cell potential in low oxygen as determined by CRA_{cell} assay.
Murine MEL cells were incubated at 0.1% oxygen (LC1; $3x10^5$ cells/ml) and treated (circle) or not (square) at day 5 of incubation with 5-fluorouracil (100 μg/ml). At day 7 of LC1, cells were washed free of drug and transferred to LC2 ($3x10^4$ cells/ml). LC2 were then incubated in air for the indicated times (days) and total number of viable cells counted. Redrawn from data reported in [32].

cle) that, once LC2 repopulation started, was identical to that of untreated LC1 cells. This means that the MEL cell population not only undergoes the process of dynamic stem cell maintenance we referred to above, but also includes a small subset of quiescent LSC, which are thereby resistant to cycle-specific antiblastic drugs [32]. This LSC subset is commonly believed to sustain minimal residual disease.

Comprehensive data relative to LSC maintenance in low oxygen as determined by CRA assays for a number of leukaemic primary cell populations and cell lines are summarized in Table **2** [see table for references]. Main information which can be driven from Table **2** is: (a) the LSC maintenance value (0.74) estimated for MEL cells by CRA_{cell} assay was close to the average value (0.81) obtained for normal murine haematopoiesis by pooling CRA_{CFC} and MRA_{CFC} data, but markedly different from the average value (0.29) of pooled CRA_{cell} and MRA_{cell} data (reported in Table **1**); (b) strikingly similar LSC maintenance values were obtained for the K562 Chronic Myeloid Leukaemia (CML) cell line by CRA_{cell} or CRA_{CFC} assays (lines 5 *vs* 6); (c) very similar LSC maintenance values were obtained for MEL cells by CRA_{cell} or *extended*CRA_{cell} assays (lines 9 *vs* 10). A reasonable explanation of all above is that the LSC compartment is functionally more homogeneous than the HSC compartment, which indeed responds quite differently to low oxygen if determined by CRA_{cell} or CRA_{CFC} assays. The conclusion that the leukaemic stem cell hierarchy is different from that of normal haematopoiesis is in keeping with current views on the issue. The higher probability to cycle of LSC compared with HSC evidenced by 5FU experiments may represent a developmental advantage of leukaemic over normal haematopoiesis. On the basis of all above, the CRA_{cell} assay was adopted as the standard method to determine LSC maintenance.

USE OF CRA_{CELL} ASSAY TO DISCRIMINATE BETWEEN LEUKAEMIA STEM AND PROGENITOR CELLS

Studies carried out with CML cells showed that the CRA_{cell} assay is capable to discriminate between LSC and Leukaemia Progenitor Cells (LPC). In these studies, the effects of low oxygen on the maintenance of LSC or LPC were evaluated together with those on the expression of *BCR/abl* oncogene product ($BCR/Abl_{protein}$), responsible for the pathogenesis of disease. The CRA_{cell} of three different stabilized CML cell lines was almost fully maintained at day 7 of incubation in low-oxygen LC1 (Table **2**), when $BCR/Abl_{protein}$, as detected by immuno-blotting, was completely suppressed (Fig. **4**), in keeping with previous findings [33-36]. However, *BCR/abl* mRNA was not suppressed in these cells, indicating that CML cells adapted to low oxygen are independent of BCR/Abl for their maintenance in culture but remain genetically leukaemic. Indeed, when these cells are transferred to growth-permissive LC2 incubated in air, they repopulate cultures with $BCR/Abl_{protein}$-expressing cells. Thus, CML cell adaptation to low oxygen is paralleled by reversible $BCR/Abl_{protein}$ suppression. As a consequence of that, the CRA_{cell} of cells adapted to low oxygen is completely insensitive to the CML inhibitor Imatinib-mesylate (IM; Gleevec®), the current first-choice drug for CML therapy, as its molecular target is suppressed. Accordingly, full sensitivity to IM is rescued in the progeny of cells adapted to low-oxygen LC1 in the course of their expansion in air-incubated LC2 [34]. These results were the first to link suppression of $BCR/Abl_{protein}$ and insensitivity to IM to the capacity of LSC of CML to adapt to the typical "hypoxic" stem cell niche environment. The question of the metabolic adaptation of LSC as well as HSC to low oxygen is beyond the scopes of this review.

The possibility to adapt the CRA_{cell} assay to discriminate between LSC and LPC of CML actually emerged in a later study where we deepened the relationship of $BCR/Abl_{protein}$ expression to CRA [35]. In this study, cells were rescued from LC1 at different times of incubation in low oxygen (Table **2**) when $BCR/Abl_{protein}$ was still fully expressed (day 7), just suppressed (day 10), or was long suppressed (day 14). Cell transfer to LC2 at day 14 of LC1 resulted in CRA_{cell} maintenance values within the range expected from the other studies carried out with MEL or CML cells. On the contrary, cells transferred to LC2 at earlier times scored much higher LC2 repopulation values, which is reasonably explained as the effect of a more (day 7) or less (day 10) promptly available BCR/Abl-dependent proliferative signaling upon cell transfer to growth-permissive conditions in normoxic LC2. These results, taken together with all previous information available, led to the conclusion that LC2 repopulation actually defines the maintenance of LSC only when $BCR/Abl_{protein}$ is long suppressed in LC1 ("selection" of LSC), while LC2 repopulation operated by $BCR/Abl_{protein}$-

Table 2. Maintenance of Leukaemia Stem/Progenitor Cell Potential in Low Oxygen as Determined by CRA Assays.

	cells	days in LC1 (0.1% O_2)	days in LC2 (peak)	assay	maintenance of LSC at 0.1% O_2	ref.
1	CD34+	8	7	CRA_{CFC}	**1.18**	[33]
2	CD34+ CML				**1.01**	
3	CD34+		21		**1.18**	
4	CD34+ CML				**0.29**	
5	K562 CML	7	14	CRA_{CFC}	**1.04**	[34]
6	K562 CML			CRA_{cell}	0.94	
7	KCL22 CML				0.88	
8	LAMA84 CML				0.93	
9	Friend's MEL	7	8	CRA_{cell}	0.74	[32]
10			18	e-CRA_{cell}	0.69	
11	K562 CML	7	15	CRA_{cell}	**1.77**	[35]
12		10	18		**1.47**	
13		14	21		**0.87**	
14	K562 CML	7	12	CRA_{cell}	**0.91**	[36]

See text and referenced papers for details and explanation. Lines 1-4: primary cells from CML patients. Lines 5-14: cells from stabilized leukaemia cell lines. Lines 11-13: at difference with all other studies, a relatively low time-0 cell density was used ($3x10^4$ cells/ml instead of $3x10^5$ cells/ml), which explains the longer incubation times (14 days) necessary for cell selection in low oxygen and, conversely, the unusually high LSC maintenance values obtained for day 7 and day 10 LC1 (lines 11 and 12). Data derive from the ratio of peak value in LC2 established with cells transferred from LC1 incubated in low oxygen to the peak value in LC2 established with cells transferred from control LC1 incubated in air. e-CRA_{cell}: *extended*CRA_{cell} Data printed in bold refer to previously unpublished calculations. In collaboration with Prof. Vincent Praloran and coworkers, Laboratoire d'Hématopoïèse Normale et Pathologique, Université Victor Segalen - Bordeaux-2, and Prof. Zoran Ivanović and coworkers, Etablissement Français du Sang - Aquitaine-Limousin, Bordeaux, France.

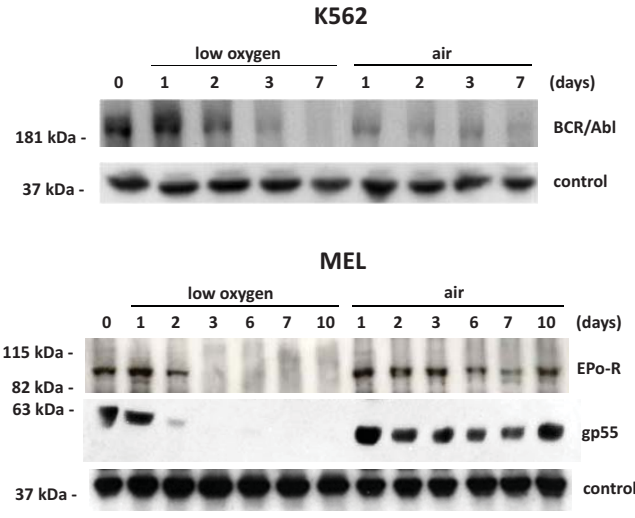

Fig. (4). Suppression of oncogenetic proteins in leukaemia cells in low oxygen.
K562 chronic myeloid leukaemia (upper panels) or MEL (lower panels) cells were plated at $3x10^5$ cells/ml, incubated in air or low oxygen and lysed at the indicated times. Lysates were subjected to immunoblotting using, for K562 cells, an anti-c-Abl antibody (Cell Signaling, cat. no. 2862), or, for MEL cells, an anti-EPo-R antibody (Santa Cruz Biotechnology, M-20, cat. no. sc-697) or a goat anti-R-MuLV gp70 antiserum (kindly provided by Dr. Sandra Ruscetti, Laboratory of Cancer Prevention, National Cancer Institute, Frederick, MD, U.S.A.). Sample loading equalization was verified on the same membranes, after stripping, using a rabbit anti-ERK1 antibody (Santa Cruz, cat. no. sc-93). Unpublished data obtained in collaboration with Dr. Serena Giuntoli and Dr. Valentina Barbetti, formerly at the Department of Experimental Pathology & Oncology of Università degli Studi di Firenze, Florence, Italy.

expressing cells refers to an LPC subset of CML population which is capable, like LSC, to stand incubation in low oxygen. Such a behaviour of LPC is not surprising, as $BCR/Abl_{protein}$ expression is linked to the constitutive induction of hypoxia-inducible factors [37]. Thus, provided $BCR/Abl_{protein}$ expression and time of incubation in low oxygen are kept under control in LC1, the CRA_{cell} assay is suitable to resolve the maintenance of $BCR/Abl_{protein}$-negative LSC from that of $BCR/Abl_{protein}$-expressing LPC.

Once the experiment (which takes place in LC1) has been carried out properly as described above, the criterion to discriminate between LSC and LPC by CRA_{cell} assay is based on the kinetics, rather than peak value, of LC2 repopulation (Fig. **5**). When cells are transferred to LC2 after selection of $BCR/Abl_{protein}$–negative LSC, LC2 repopulation follows a kinetics characterized by a ~10 day-long initial lag-phase (solid line), reminiscent of that of *extended*-CRA_{cell} of 5FU-treated MEL cells (Fig. **3**). On the contrary, when $BCR/Abl_{protein}$ is fully expressed in LC1 at the time of transfer, LC2 are repopulated rapidly, with a kinetics lacking the lag-phase (dotted line). An "intermediate" LC2 repopulation kinetics is obtained (dashed line) with cells rescued from LC1 immediately upon suppression of $BCR/Abl_{protein}$ (as estimated by western blotting). We concluded that the absence or the presence of a ~10 day-long lag-phase during LC2 repopulation reflects whether LSC have been selected or not in low-oxygen LC1 at the time of transfer. When this selection occurs, the entity of LC2 repopulation is markedly

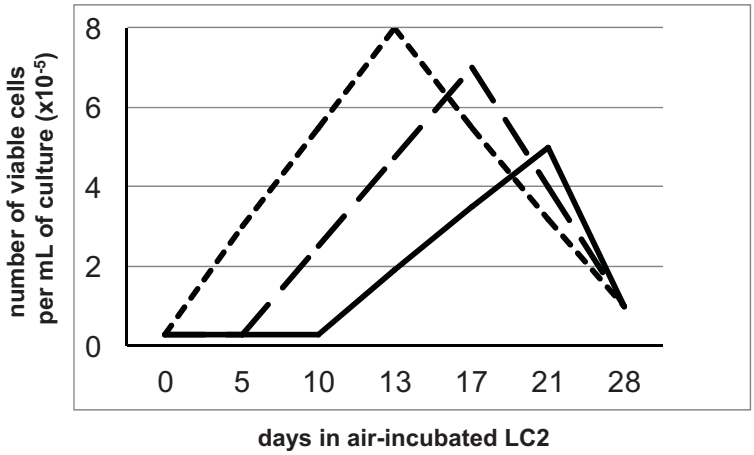

Fig. (5). CRA_{cell} assay of chronic myeloid leukaemia cells incubated in low oxygen for different times.
Low-density cultures ($3x10^4$ cells/ml) of K562 cells were incubated in low oxygen (LC1) for 7 (dotted line), 10 (dashed line) or 14 (solid line) days and then transferred at $3x10^4$ cells/ml to LC2. LC2 were incubated in air for the indicated times (days) and total number of viable cells counted. Schematic representation of pooled data from references [35, 36], as well as unpublished work. In collaboration with Dr. Michele Tanturli, Department of Clinical Pathophysiology, formerly at the Department of Experimental Pathology & Oncology, Università degli Studi di Firenze, Florence, Italy.

lower that that obtainable by transferring unselected cells. In summary, these studies pointed to the loss of $BCR/Abl_{protein}$ expression in LC1 as a molecular marker of LSC selection in low oxygen, and to the type of repopulation kinetics in LC2 as a functional indicator of this selection.

The suppression of oncogenetic proteins other than $BCR/Abl_{protein}$ in low oxygen has been also observed. Fig. **4** shows that incubation in low oxygen inhibits the expression of erythropoietin receptor (EPo-R) as well as the retroviral envelope protein-encoding gene product gp55, which are involved in MEL oncogenesis. It is worth pointing out that EPo-R and gp55 suppression is complete (day 3) well before the time of LC1 cell transfer to LC2 (day 7) in the experiments of Fig. **3**. The timing between suppression of the oncogenetic protein in LC1, as estimated by western blotting (the sensitivity of which may be insufficient to detect a residual expression of the oncogenetic protein) and transfer to LC2 is indeed critical to ensure stem cell selection in LC1 (Fig. **5**). The functional role of suppression of oncogenetic proteins during LSC selection may be hypothesized to rely on the fact that an "excess" of growth-promoting signals may antagonize stem cell self-renewal and maintenance, as it was verified in some cases for normal haematopoiesis (Table **1**) [27, 28].

THE "6th WAY" to IM RESISTANCE OF CML, AS REVEALED BY CRA ASSAY

When low-oxygen LC1 are treated with IM after CML cells have been selected appropriately to repopulate LC2 with a ~10 day-long lag-phase, this repopulation is unaffected by the drug treatment, indicating that selected cells are insensitive to the drug. These results have been confirmed using primary cells explanted from CML patients [35]. Selection in low oxygen of LSC where $BCR/Abl_{protein}$ is completely suppressed [34, 35] impacts on current knowledge about IM resistance of CML cells as follows. Five mechanisms, BCR/Abl-dependent or -independent, are traditionally believed to determine resistance to IM: (a) mutations of *BCR*/*abl* gene in tyrosine kinase domain affecting IM binding; (b) amplification of *BCR*/*abl* gene; (c) secondary mutations outside *BCR*/*abl* gene determining BCR/Abl-independent survival and proliferation (mutation-driven loss of "oncogene addiction") [38]; (d) enhanced activity of drug exporters; (e) quiescence. Our studies led to identify a novel mechanism (f): environment-enforced $BCR/Abl_{protein}$ suppression, arguably coupled to LSC homing within the "hypoxic" stem cell niches and not necessarily linked to quiescence (see below).

Mechanism (f) predicts that *all* LSC (being *all* LSC selectable in low oxygen), but *none* of LPC, exhibit "primary resistance" (more appropriately referred to as "refractoriness") to IM due to the lack of its molecular target. An advantage of mechanism (f) is its simplicity, *i.e.* that it does not need, to explain IM resistance, secondary genetic events affecting a subset of CML cells to be postulated. Indeed, the refractoriness to IM of low oxygen-selected LSC is not genetically blocked, but fully reversible, provided LSC moves to a tissue area where $BCR/Abl_{protein}$ can be re-expressed.

WHAT SELECTION IN LOW OXYGEN AND REFRACTORINESS TO IM ALLOW TO INFER ABOUT LSC OF CML

In low oxygen, LSC are apparently forced to suppress the BCR/Abl-dependent oncogenetic signalling, which would confer upon LSC themselves a growth advantage over normal HSC. $BCR/Abl_{protein}$–negative LSC are deprived of this advantage, but not of physiological properties of HSC, including the capacity to cycle at low oxygen tensions we described above. In low oxygen, in other words, LSC of CML return to a HSC-like phenotype, exhibiting no oncogene addiction [38]. However, cycling of LSC in low oxygen, given their leukaemic genotype, sustains not only dynamic stem cell maintenance, but also neoplastic progression, as transmission of mutations to progeny relies on cell cycling. Such LSC cycling, as it is likely coupled to self-renewal but not clonal expansion which is restrained in low oxygen [27], results in the long-term maintenance of subclinical, yet progressing, disease. $BCR/Abl_{protein}$ suppression in low oxygen is therefore better suited than LSC quiescence to explain the combination of refractoriness to IM with liability to neoplastic progression.

On the basis of all above, the refractoriness to IM of LSC selected in low oxygen appears to be an adaptive feature which, under metabolic pressure in tissue environment, is flexibly and reversibly enforced in a subset of cells. This phenomenon is very well in keeping with the *Chiaroscuro* model of reversible transition between the stem and progenitor cell phenotypes proposed for normal haematopoiesis [39]. We discussed extensively elsewhere the relevance of this model to the interpretation of our experimental data [35], as well as the possible mechanisms of modulation of $BCR/Abl_{protein}$ expression [35, 36]. However, it is worth stressing here that our results allow to address the modelling of leukaemic "stemness" according to a new perspective. Two alternative models have been proposed for cancer cell "stemness": the *stem cell in normal stem cell* and the *stem cell in progenitor cell* models [40]. We propose both models to reflect CML biology, being the former adequate to

describe BCR/Abl$_{protein}$-negative LSC and the latter LSC undergoing expression of BCR/Abl$_{protein}$ (Fig. **6**).

THE "ISCHEMIC" STEM CELL NICHE HYPOTHESIS

The context-specific fluctuations of BCR/Abl$_{protein}$ expression we described were deepened by varying time-0 cell density and glucose concentration in low-oxygen LC1 [35]. At not-limiting residual glucose concentrations in culture medium, BCR/Abl$_{protein}$ expression survives a fairly long incubation, while cell population expands significantly, in spite of being in low oxygen. Is indeed glucose exhaustion in LC1, rather than time of incubation in low oxygen, the driving force of BCR/Abl$_{protein}$ suppression, which determines the pattern of LC2 repopulation shown in Fig. **5**. There is no need to imagine the existence *in vivo* of tissue areas where glucose diffusion is permanently insufficient, resembling ischemia rather than hypoxia. We hypothesize "ischemic" stem cell niches to be established within "hypoxic" stem cell niches where glucose gets frequently close to exhaustion as a consequence of the combination of scarce diffusion with the enhanced consumption due to the Pasteur effect typical of cell metabolism in low oxygen. The functional relationship of the "ischemic" to the "hypoxic" stem cell niche in CML is also shown in Fig. **6**. We hypothesize the former to be established within the very core of the latter and to represent the site of quiescence or BCR/Abl-independent self-renewal. On the other hand, moving from these sites to neighbouring low-oxygen areas where glucose concentration is higher, LSC would be recruited to BCR/Abl$_{protein}$ expression and thereby generate BCR/Abl$_{protein}$-expressing LPC capable to drive clonal expansion.

USE OF CRA$_{CELL}$ ASSAY TO TEST DRUGS POTENTIALLY ACTIVE ON LSC OF CML

We proposed an *in vitro* pre-screening strategy for LSC-targeting drugs based on CML cell incubation in low oxygen prior to drug treatment, in order to (a) suppress BCR/Abl$_{protein}$ and select cells with the IM-resistant LSC phenotype, (b) mimic the most likely scenario *in vivo* where LSC are already placed in the "hypoxic" stem cell niches before therapy starts. An example of how this strategy is applied is provided by a study where we tested the proteasome inhibitor Bortezomib (BTZ) on CML cells of the K562 line, determining its effects separately on LSC and LPC [36]. Cells from day-2 low-oxygen LC1, due to the maintained BCR/Abl$_{protein}$ expression, rapidly repopulated LC2, to peak at day 10. LC2 repopulation was totally suppressed by BTZ addition to LC1 at time 0, but resulted completely resistant to that at day 1. This indicates that LPC are highly sensitive to BTZ, but are capable to get protected from the effects of BTZ following a one-day adaptation to low oxygen. LC2 repopulation by cells selected in day-7 LC1 was delayed, starting from day 7 to peak at day 21. In this case, BTZ significantly, but partially, reduced LC2 repopulation, irrespective of its addition to LC1 at time 0 or day 1. This means that the K562 cell line contains, from the beginning of incubation, a cell subset predisposed to exhibit an LSC phenotype and to undergo selection in low oxygen, and that this subset is in part BTZ-resistant *per se*, *i.e.* before selection. When the experiment were repeated using primary cells explanted from CML patients, LSC resulted resistant to either time-0 or day-1 BTZ. These data are predictive of a failure of attempts to use BTZ to suppress IM-resistant CML cells and prevent minimal residual disease [41], suggesting the outcome of these attempts to be evaluated with caution and made object of further deepening. Taken together, these results indicate that profound differences of drug response exist between LSC and LPC of CML cells and that the response of individual subsets can be tested separately by using (a) stabilized cell lines, (b) cell incubation and selection in low oxygen and (c) the CRA$_{cell}$ assay. The parallel use of different cell lines of the same kind would overcome cell line-specific responses and thereby increase the reliability of results.

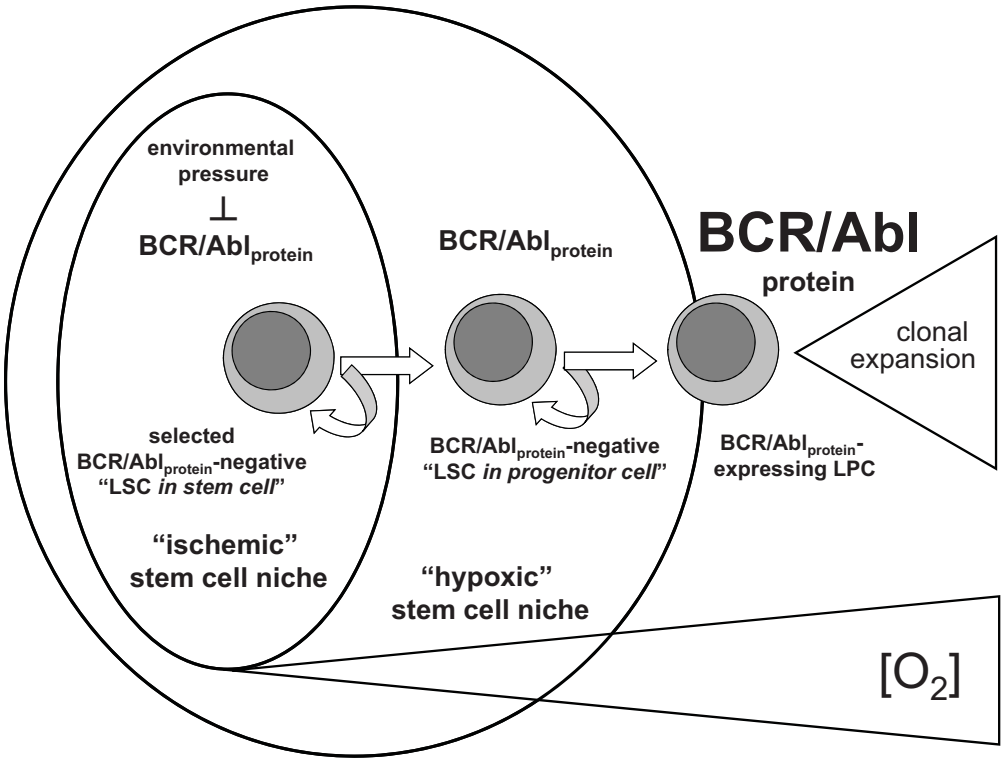

Fig. (6). Stem cell niche(s) and the immature CML cell subsets which can be distinguished by CRA$_{cell}$ assay.
Schematic representation of the effects of environment on BCR/Abl$_{protein}$ expression and the CML stem/progenitor cell hierarchy. LSC, leukaemia stem cells; LPC, leukaemia progenitor cell; "ischemic" stem cell niche, tissue area at low glucose/low oxygen; "hypoxic" stem cell niche, tissue area at low oxygen. Note that in "hypoxic" stem cell niche, according to this hypothesis, BCR/Abl expression is allowed but LSC remains BCR/Abl$_{protein}$-negative.

USE OF CRA_{CELL} ASSAYS TO TEST DRUGS ON LSC OR LPC OF CML: A PRACTICAL SUMMARY

The response of LSC or LPC of CML to drug treatment can be easily tested separately by CRA_{cell} assay, according to the following simple procedure. (A) Add the drug to LC1 incubated in low oxygen when $BCR/Abl_{protein}$ is still fully expressed (to determine the effects on LPC) or long after its suppression (to determine the effects on LSC). (B) Rescue cells from LC1 at the end of the appropriate treatment time. (C) Wash LC1 cells free of drug. (D) Transfer LC1 cells to LC2 to be incubated in air. (E) Determine the LC2 repopulation pattern (peak levels and kinetics) by counting the total number of viable cells. (F) Evaluate the effects of drug treatment by comparing this pattern with that of LC2 repopulation driven by cells rescued from control untreated LC1. Step (A) can be complicated with the addition of 5-FU long after $BCR/Abl_{protein}$ suppression, cell wash and administration of the drug to be tested, when one wants to estimate its effects on *extended*CRA_{cell}. From the methodological point of view, it is worth clarifying here the reasons for using low-oxygen LC1 to study $BCR/Abl_{protein}$-expressing LPC, which do not undergo selection in low oxygen. First, as low oxygen is used to select LSC, the rescue of LPC too from low-oxygen LC1 warrants that LPC and LSC are maintained under similar conditions as long as possible before estimating their CRA. Second, low oxygen suppresses the expansion of cell bulk (see Fig. **1**) and minimizes the overall loss of proliferation potential in culture, including that of LPC, resulting in a "cleaner" kinetics of LC2 repopulation. Third, all CML cells which are capable to stand low oxygen, including LPC, are worth to be targeted to prevent relapse of disease.

CONCLUDING REMARKS

We were the first to propose to select normal or leukaemic stem/progenitor cells in low oxygen as a pre-condition to their characterization. Such an approach exploited the concept of cancer as a "hypoxia"-related disease [42], where (pathological) hypoxia enforces a cell response which is fundamental to the long-term maintenance of disease, *i.e.* minimal residual disease. In the course of these studies, we developed the CRA assays as a simple and economic, yet accurate and reliable, method to measure the maintenance of stem/progenitor potential within a normal or neoplastic cell population. The combination of CRA assays with cell incubation at low oxygen enables to discriminate among different hierarchical levels within the tissue regeneration machinery, which is especially useful to test the effects of cytostatic or cytotoxic drugs on individual immature cell subsets composing this machinery. Thus, when drug effects need to be evaluated on this ground, the CRA assays are in our opinion of particular value for an *in vitro* pre-screening strategy to be adopted systematically before drugs are tested *in vivo*, where not only short-term, but also long-term repopulating stem cells can be assayed. Overall, we believe the development of CRA assays to represent a good example of "translational" work between basic science and applied pharmacology.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

Research was supported by the following sources of funding: (a) Istituto Superiore di Sanità (ISS) - National Programme "Stem Cells" (grant # CS64); (b) Istituto Toscano Tumori (ITT); (c) Associazione Italiana per la Ricerca sul Cancro (AIRC); (d) Ministero della Salute (grant # RF-TOS-2008-1163728); (e) Regione Toscana – Programma per la Ricerca in Materia di Salute; (f) Associazione Italiana per la Lotta contro le Leucemie e i Linfomi (AIL; Sezione di Prato); (g) Fondazione Cassa di Risparmio di Volterra; (h) Fondazione Oretta Bartolomei-Corsi. ER was supported by Federazione Italiana per la Ricerca sul Cancro (FIRC) for the years 2001-2003 and 2007-2008. The important intellectual contribution of professor Massimo Olivotto (Department of Experimental Pathology & Oncology of Università degli Studi di Firenze) is also acknowledged. MGC designed and performed research and analyzed data; ER performed research, analyzed data and revised the paper; PDS designed research, analyzed data and wrote the paper.

GLOSSARY & ABBREVIATIONS

New or Less New Concepts Referred to

1) "dynamic" stem cell maintenance
2) "hypoxic" stem cell niche
3) "ischemic" stem cell niche
4) oncogene addiction
5) stem/progenitor cell potential

Stem/Progenitor Cell Assays Mentioned

1) competitive repopulation assay
2) Culture-Repopulating Ability (CRA) assays
3) CRA_{cell} assay
4) CRA_{CFC} assay
5) Delta assays
6) Marrow-Repopulating Ability (MRA) assays
7) MRA_{cell} assay
8) MRA_{CFC} assay
9) $SCID_{mice}$-Repopulating Cell (SRC) assay

Other Abbreviations

1) BCR/Abl protein ($BCR/Abl_{protein}$)
2) Colony-Forming Cell (CFC)
3) Chronic Myeloid Leukaemia (CML)
4) Haematopoietic Stem Cell (HSC)
5) Imatinib-Mesylate (IM)
6) Leukaemia Progenitor Cell (LPC)
7) Leukaemia Stem Cell (LSC)
8) Murine Friend's Erythro-Leukaemia (MEL)
9) pre-CFC
10) primary liquid culture (Liquid Culture 1; LC1)
11) secondary liquid culture (Liquid Culture 2; LC2)

REFERENCES

[1] Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 1961; 14: 213-22.
[2] Siminovitch L, McCulloch EA, Till JE. The distribution of colony-forming cells among spleen colonies. J Cell Physiol 1963; 62: 327-36.
[3] Bradley TR, Metcalf D. The growth of mouse bone marrow cells *in vitro*. Austr J Exp Biol Med Sci 1966; 44: 287-99.
[4] Pluznik DH, Sachs L. The induction of clones of normal mast cells by a substance from conditioned medium. Exp Cell Res 1966; 43: 553-63.
[5] Bradley TR, Sumner MA. Stimulation of mouse bone marrow colony growth *in vitro* by conditioned medium. Austr J Exp Biol Med Sci 1968; 46: 607-18.
[6] Sumner MA, Bradley TR, Hodgson GS, Cline MJ, Fry PA, Sutherland L. The growth of bone marrow cells in liquid culture. Br J Haematol 1972; 23:221-34.
[7] Allen TD, Dexter TM. Cellular interrelationships during *in vitro* granulopoiesis. Differentiation 1976; 6: 191-4.
[8] Bock TA. Assay systems for hematopoietic stem and progenitor cells. Stem Cells 1997;15 Suppl 1: 185-95.

[9] Dello Sbarba P, Cipolleschi MG, Olivotto M. Hemopoietic progenitor cells are sensitive to the cytostatic effect of pyruvate. Exp Hematol 1987;15:137-42

[10] Moore MAS. Clinical implications of positive and negative hematopoietic stem cell regulators. Blood 1991; 78: 1-19.

[11] Gordon MY. Human haemopoietic stem cell assays. Blood Rev 1993; 7: 190-7.

[12] Moore MAS, Hoskins I: *Ex vivo* expansion of cord blood-derived stem cells and progenitors. Blood Cells 1994; 20: 468-79.

[13] Shapiro F, Tzy-Jyun Y, Moskowitz C, *et al*. Effects of prior therapy on the *in vitro* proliferative potential of stem cell factor plus filgrastim-mobilized CD34-positive progenitor cells. Clin Cancer Res 1997; 3: 1571-8

[14] Cipolleschi MG, Dello Sbarba P, Olivotto M. The role of hypoxia in the maintenance of hematopoietic stem cells. Blood 1993; 82: 2031-7.

[15] Traycoff CM, Cornetta K, Yoder MC, Davidson A, Srour EF. *Ex vivo* expansion of murine hematopoietic progenitor cells generates classes of expanded cells possessing different levels of bone marrow repopulating potential. Exp Hematol 1996; 24: 299-306.

[16] Ploemacher RE, Brons RH. Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence for a pre-CFU-S cell. Exp Hematol 1989; 17: 263-6.

[17] Ivanović Z, Bartolozzi B, Bernabei PA, *et al*. Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors. Br J Haematol 2000; 108: 424-9.

[18] Cipolleschi MG, D'Ippolito G, Bernabei PA, *et al*. Severe hypoxia enhances the formation of erythroid bursts from human cord blood cells and the maintenance of BFU-E *in vitro*. Exp Hematol 1997; 25: 1187-94.

[19] Ivanović Z, Dello Sbarba P, Trimoreau F, Faucher JL, Praloran V. Primitive human HPCs are better maintained and expanded *in vitro* at 1 percent oxygen than at 20 percent. Transfusion 2000; 40: 1482-8.

[20] Danet GH, Pan Y, Luongo JL, Bonnet DA, Simon MC. Expansion of human SCID-repopulating cells under hypoxic conditions. J Clin Invest 2003; 112: 126-35.

[21] Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 1978; 4: 7-25.

[22] Takubo K, Suda T. Roles of the hypoxia response system in hematopoietic and leukemic stem cells. Int J Hematol 2012; 95: 478-83.

[23] Eliasson P, Jönsson JI. The hematopoietic stem cell niche: low in oxygen but a nice place to be. J Cell Physiol 2010; 222: 17-22.

[24] Ivanovic Z. Hypoxia or in situ normoxia: the stem cell paradigm. J Cell Physiol. 2009; 219: 271–5.

[25] Guitart V, Hammoud M, Dello Sbarba P, Ivanovic Z, Praloran V. Slow-cycling/quiescence balance of hematopoietic stem cells is related to physiological gradient of oxygen. Exp. Hematol. 2010; 38: 847-51.

[26] Cipolleschi MG, Rovida E, Ivanović Z, Praloran V, Olivotto M, Dello Sbarba P. The expansion of murine bone marrow cells preincubated in hypoxia as an *in vitro* indicator of their marrow-repopulating ability. Leukemia 2000; 14: 735-9.

[27] Ivanović Z, Belloc F, Faucher JL, Cipolleschi MG, Praloran V, Dello Sbarba P. Hypoxia maintains and interleukin-3 reduces the pre-colony-forming cell potential of dividing CD34(+) murine bone marrow cells. Exp Hematol 2002; 30: 67-73.

[28] Ivanović Z , Hermitte F, Brunet de la Grange P, *et al*. Simultaneous maintenance of human cord blood SCID-repopulating cells and expansion of committed progenitors at low O2 concentration (3%). Stem Cells 2004; 22: 716-24

[29] Kovacević-Filipović M, Petakov M, Hermitte F, *et al*. Interleukin-6 (IL-6) and low O(2) concentration (1%) synergize to improve the maintenance of hematopoietic stem cells (pre-CFC). J Cell Physiol 2007; 212: 68-75.

[30] Hammoud M, Vlaski M, Duchez P, *et al*. Combination of low O(2) concentration and mesenchymal stromal cells during culture of cord blood CD34(+) cells improves the maintenance and proliferative capacity of hematopoietic stem cells. J Cell Physiol 2012; 227: 2750-8.

[31] Pastrana E, Silva-Vargas V, Doetsch F. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. Cell Stem Cell 2011; 8: 486-98.

[32] Giuntoli S, Rovida E, Gozzini A, *et al*. Severe hypoxia defines heterogeneity and selects highly immature progenitors within clonal erythroleukaemia cells. Stem Cells 2007; 25: 1119-25.

[33] Desplat V, Faucher JL, Mahon FX, Dello Sbarba P, Praloran V, Ivanovic Z. Hypoxia modifies proliferation and differentiation of CD34(+) CML cells. Stem Cells 2002; 20: 347-54.

[34] Giuntoli S, Rovida E, Barbetti V, Cipolleschi MG, Olivotto M, Dello Sbarba P. Hypoxia suppresses BCR/Abl and selects Imatinib-insensitive progenitors within clonal CML population. Leukemia 2006; 20: 1291-3.

[35] Giuntoli S, Tanturli M, Di Gesualdo F, Barbetti V, Rovida E, Dello Sbarba P. Glucose availability in hypoxia regulates the selection of Chronic Myeloid Leukaemia progenitor subsets with different resistance to Imatinib-mesylate. Haematologica 2011; 96: 204-12.

[36] Tanturli M, Giuntoli S, Barbetti V, Rovida E, Dello Sbarba P. Hypoxia selects bortezomib-resistant stem cells of chronic myeloid leukemia. PLoS-One 2011; 6(2):e17008.

[37] Mayerhofer M, Valent P, Sperr WR, Griffin JD, Sillaber C. BCR/ABL induces expression of vascular endothelial growth factor and its transcriptional activator, hypoxia inducible factor-1alpha, through a pathway involving phosphoinositide 3-kinase and the mammalian target of rapamycin. Blood 2002; 100: 3767-75.

[38] Felsher DW. Reversing cancer from inside and out: oncogene addiction, cellular senescence, and the angiogenic switch. Lymphat Res Biol 2008; 6: 149-54.

[39] Quesenberry PJ, Colvin GA, Lambert JF. The chiaroscuro stem cell: a unified stem cell theory. Blood 2002; 100: 4266-71.

[40] Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001; 414: 105-11.

[41] Santos FP, Kantarjian H, McConkey D, *et al*. Pilot study of bortezomib for patients with imatinib-refractory chronic myeloid leukemia in chronic or accelerated phase. Clin Lymphoma Myeloma Leuk 2011; 11: 355-60.

[42] Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 2007; 26: 225-39.

Received: December 26, 2012 **Accepted: February 1, 2013**